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# **The Effect of Lactic Acid on Mast Cell Function**

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science  
in Biology at Virginia Commonwealth University

By

Andrew Jason Spence

Bachelor of Science in Biology

Virginia Commonwealth University, 2012

Director: John J. Ryan, Ph.D.,  
Professor of Biology

Virginia Commonwealth University

Richmond, Virginia

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## Table of Contents

List of Figures	iv
List of Abbreviations	v
Abstract	vii
Introduction	1
Materials and Methods	4
Results	9
Discussion	13
Figures	17
References	40
Vita	44

## List of Figures

Figure 1A-H: *Lactic Acid Causes Differential Cytokine Production in C57BL/6 BMMCs Depending on Activation*

Figure 2A-G: *Lactic Acid Causes Differential Cytokine Production in 129/SvJ BMMCs Depending on Activation*

Figure 3A-B: *Lactic Acid Promotes Increased VEGF Production in C57BL/6 BMMCs*

Figure 4A-B: *Lactic Acid Does Not Change IgE or IL-33 Receptor Expression*

Figure 5A-D: *Lactic Acid Promotes a G0/G1 Cell Cycle Arrest in BMMCs*

Figure 6A-D: *Lactic Acid Does Not Alter IgE-mediated Degranulation*

Figure 7A-C: *Lactic Acid Alters IL-33 Mediated Cytokine Production in a pH Dependent Manner*

Figure 8A-C: *Lactic Acid Effects on IgE-induced Cytokine Production are Not pH-dependent*

Figure 9A-E: *Lactic Acid Decreases IL-33-mediated TAK1 and JNK Phosphorylation*

Figure 10A-B: *TAK1 and JNK STIs Mimic Lactic Acids Effect on IL-33 Activation*

Figure 11: *Lactic Acid Decreases mir-155 Levels*

## List of Abbreviations

AKT- Protein kinase B (PKB), involved in glucose metabolism, apoptosis, cell proliferation, transcription, angiogenesis

BMMC- Bone marrow-derived mast cells

BHK- Supernatant from SCF producing cells

c-Kit- SCF receptor

CTL- Cytotoxic T Lymphocyte

CD 63- marker for degranulation

CD 107a- lysosomal associated membrane protein 1, marker for degranulation

DAMP- Damage Associated Molecular Pattern

DNP-HSA- Dinitrophenylated human serum albumin

FcεRI- High-affinity IgE receptor

HIF-1 $\alpha$ - Hypoxia inducible factor

IgE- Immunoglobulin E

IgG- Immunoglobulin G

IL-3- Interleukin 3, vital for mast cell survival

IL-6- Interleukin 6, a pro-inflammatory cytokine

IL-13- Interleukin 13, mimics some IL-4 properties, promotes mucus production

IL-33- Interleukin 33, a DAMP produced by stressed or injured cells, binds the ST2 receptor

JNK- c-Jun N-terminal kinases

LA- L-(+)-Lactic acid

LPS- Lipopolysaccharide

MAPK- Mitogen-activated protein kinases

MCP-1- Monocyte chemotactic protein 1

MIP-1 $\alpha$ - macrophage inflammatory protein 1  $\alpha$

NF $\kappa$ B- Nuclear factor  $\kappa$ B

p38- P38 mitogen-associated protein kinases

p65- Transcription factor 65, also known as nuclear factor NF $\kappa$ B p65 subunit

PAF- Platelet activating factor

SCF- Stem cell factor, vital for mast cell survival

TAK1-Transforming growth factor  $\beta$  activated kinase 1

TAM- Tumor associated macrophage

TME- Tumor microenvironment

TNF- Tumor necrosis factor  $\alpha$ , a pro-inflammatory cytokine

VEGF- Vascular endothelial growth factor

WEHI- Supernatant from IL-3 producing cells

# **Abstract**

## **THE EFFECT OF LACTIC ACID ON MAST CELL FUNCTION**

By Andrew Jason Spence, Bachelor of Science in Biology

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biology at Virginia Commonwealth University.

Virginia Commonwealth University, 2014

Major Director: John J. Ryan, Ph.D., Professor of Biology

This study shows for the first time the effect that L-(+)-lactic acid has on mast cell activation. Lactic acid is a byproduct of anaerobic glycolysis and is associated with inflammatory environments such as wounds, tumors and, asthma. In this study, pre-treatment with lactic acid altered cytokine production by bone marrow-derived mast cells (BMMC). Specifically, lactic acid enhanced cytokine secretion following IgE cross-linking, but decreased IL-33 mediated cytokine production. These effects were altered by genetic background, since C57BL/6 mast cells demonstrated the aforementioned result, but lactic acid had no effect on IgE-mediated cytokine production in 129/SvJ mast cells. The affected cytokines included IL-6, TNF, MCP-1, MIP-1 $\alpha$ , IL-13, and VEGF. Lactic acid pretreatment promoted a G0/G1 cell cycle arrest. Investigation into the IL-33 signaling pathway showed lactic acid decreased TAK1 and JNK phosphorylation, while increasing phosphorylated AKT levels. Blocking JNK and TAK1 with a small molecule inhibitor mimicked the effects of lactic acid. Interestingly, lactic acid significantly increased IL-33 mediated VEGF. An *in vitro* angiogenesis assay confirmed that



mast cells were pro-angiogenic in a lactic acid-rich environment. Taken together, these data show that lactic acid impacts mast cell function, possibly promoting a pro-angiogenic, anti-inflammatory phenotype.

## Introduction

Mast cells are sentinels of the innate immune system, guarding the body against invading bacterial and parasitic infections. However, mast cells are best known for the major role they play in Type II hypersensitivities such as allergy and asthma. The binding of a harmless allergen to mast cell-bound IgE, and the subsequent signaling through the IgE receptor, FcεRI, result in a signaling cascade that forces the mast cell to release both early and late phase mediators (1-5). The early phase mediators, released within minutes of activation, include tryptases, chymases, histamine, prostaglandins, leukotrienes and Platelet Activating Factor (PAF), while the late phase mediators, released hours later, consist of IL-4, IL-5, IL-6, IL-10, IL-13, TNF, MIP-1α (1-5).

These factors produce the clinical symptoms of immediate hypersensitivity, including the skin wheal-and-flare response, itching, and vasodilation/edema. Mast cells can drive chronic disease such as asthma upon repeated exposure to the same allergens, which results in airway remodeling due to chronic inflammation mast cells cause (1-5).

IgE crosslinking is not the only way mast cells are activated. IL-33 is a recently discovered alarmin in the IL-1 family produced by endothelial cells, epithelial cells, fibroblasts, and keratinocytes in response to damage or stress and promotes a TH2 response (6-10). Mast cells are one of the few cells in the body known to have the IL-33 receptor, ST2 (11). IL-33 binding by mast cells results in the release of cytokines, chemokines and lipid mediators (6,7). IL-33 has also been shown to promote mast cell survival, maturation and adhesion (8,9,12). While IL-33 has been shown to be a poor inducer of degranulation, it augments degranulation triggered through the IgE receptor (6, 7). IL-33 has both protective and harmful effects. From a protective standpoint, IL-33 has beneficial effects in atherosclerosis, cardiac remodeling and

helminth infection (8). From an autoimmune standpoint, IL-33 has been linked to asthma, rheumatoid arthritis, multiple sclerosis, Type I diabetes, and skin inflammation (7, 13-23).

Inflammatory environments have specific changes in their microenvironments (9). A well-known example of this is the tumor microenvironment (TME). Tumors are known to preferentially undergo anaerobic glycolysis, even in the presence of sufficient oxygen, resulting in a hypoxic and lactic acid rich microenvironment with a low pH (10-16). These unique environmental factors can alter cellular responses, allowing tumors to escape immune surveillance (14, 17-19). There is sufficient evidence that the TME promotes tumor-associated macrophages (TAM) to take on an M2 phenotype, which is anti-inflammatory, and has reduced antigen presentation ability (11, 19). Tumor-derived lactic acid has also been shown to inhibit dendritic cell function, resulting in decreased proliferation and reduced antigen presentation (12). Among cytotoxic T cells, lactic acid decreases proliferation and inhibits their cytotoxic function (14). Other examples of altered microenvironments that have increased lactate levels are obesity, hypertension and Type II diabetes, as well as tissues suffering injury, infection, inflammation or ischemia (17, 18, 20, 21).

Mast cells are found in nearly all tissues except blood and can have phenotypic plasticity altered by microenvironmental factors, including SCF, IL-10, and TGF $\beta$ 1 (22-25). Mast cells are also known to be one of the first cell types to invade into a developing tumor (26). The pro- or anti-tumorigenic role of mast cells has been argued either way (26, 27). The ability of mast cells to produce heparin, IL-8, VEGF, histamine, mitogenic factors, such as platelet-derived growth factor (PDGF), nerve GF (NGF), SCF, and proteases, collectively contribute to tumor survival, angiogenesis, metastasis, and escape from immune surveillance (27). However, mast cells can produce IL-1, IL-4, IL-6, and TNF that can induce tumor cell apoptosis, tryptase, which

stimulates protease-activated receptors and induces inflammation, and chondroitin sulfate, which could act as a decoy and inhibit metastases (27). Considering that lactic acid is known to alter cellular responses in inflammatory environments, and that lactic acid and mast cells are coming into direct contact in the body, an investigation into lactic acid effects on mast cell function is warranted.

## Materials and Methods

### *Animals*

C57BL/6 and 129/SvJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used at a minimum of 6 weeks old, with approval from the Virginia Commonwealth University institutional animal care and use committee (IACUC).

### *Mouse Mast Cell Cultures*

Mouse bone marrow-derived mast cells (BMMCs) were derived by harvesting bone marrow aspirates from the femur of mice, followed by culture in complete RPMI (cRPMI) 1640 medium (Invitrogen Life Technologies, Carlsbad, CA) containing 10% FBS, 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1mM sodium pyruvate, and 1mM HEPES (all from Corning, Corning, NY), supplemented with IL-3-containing supernatant from WEHI-3B cells and SCF-containing supernatant from BHK-MKL cells. The final concentration of IL-3 and SCF were adjusted to 1ng/ml and 10ng/ml, respectively, as measured by ELISA. BMMC were used after 3 weeks of culture, at which point these primary populations are >90% mast cells, based on staining for c-Kit and FcεRI expression.

### *Cytokines and Reagents*

Recombinant mouse IL-3, SCF, and IL-33 cytokines as well as IL-6, TNF, and MCP-1 ELISA kits were purchased from BioLegend (San Diego, CA). MIP-1α and VEGF ELISA kits were purchased from PeproTech (Rocky Hill, NJ). IL-13 ELISA kits were purchased from eBioscience (San Diego, CA). L-(+)-lactic acid and DNP-HSA were purchased from Sigma-Aldrich (St. Louis, MO).

### *Cell Culture Conditions*

For IgE-mediated activation, BMMC were first cultured overnight in cRPMI containing 10ng/ml IL-3 and SCF, +/- IgE at a final concentration of 0.5 µg/ml. Cells were then washed and resuspended at  $2 \times 10^6$  cells/ml in cRPMI with 20ng/ml IL-3 and SCF. An equal volume of 25mM lactic acid in cRPMI was added to the cell suspension, resulting in a final cell concentration of  $1 \times 10^6$  cells/ml, 10ng/ml IL-3 and SCF, and 12.5mM lactic acid. Control conditions received cRPMI in place of lactic acid. After 6 hours of pretreatment in lactic acid media, cells then received 50ng/ml DNP-HSA for 16 hours, after which supernatant was collected. For IL-33 activation, cells were cultured in lactic acid as described above for 24 hours, and then stimulated with 100ng/ml of IL-33 for 16 hours, after which supernatants were collected.

### *Degranulation*

BMMCs were cultured at  $1 \times 10^6$  with IgE in either cRPMI or lactic acid media for 6 hours prior to IgE crosslinking with 50ng/mL of DNP-HSA, as described above. Cells were crosslinked for 45 minutes before being stained with CD63 and CD107a antibodies for **X** amount of time. Cells were then washed with PBS and resuspended in 200µl of FACS buffer. Samples were then run on a BD FACSCalibur (BD Biosciences).

### *BMMC Receptor Flow Cytometry*

Cells were cultured at  $1 \times 10^6$ /ml in either cRPMI or lactic acid media for 6 or 24 hours prior to staining. Afterwards, cells were washed in PBS twice, centrifuged and resuspended in FACS buffer (PBS/3% FBS/0.01% sodium azide) containing the indicated antibodies. Samples were incubated at 4°C for 30-45 minutes, washed twice with PBS, and resuspended in 200µl of FACS buffer. Samples were then analyzed using a on a BD FACSCalibur (BD Biosciences).

### *PI DNA Staining*

BMMCs were cultured at 50,000 cells/ml in variable amounts of IL-3/SCF (0/0, 1/10, 1/25, 1/50 and 10/10 respectively) with or without 12.5 mM lactic acid for 36 hours. At the end of 36 hours, cells were washed in PBS and stored in Fixation Buffer (52.5% Ethanol, 35% PBS, 12.5% FBS) for a minimum of 6 hours. Afterwards, cells were washed in PBS and stained incubated with PI Staining Buffer (PBS, RNase A, PI) for 2 hours in the dark at 4°C, followed by analysis with a BD FACSCalibur (BD Biosciences).

### *Western Blotting*

Cells were cultured at  $2 \times 10^6$ /ml and lysed in Lysis Buffer (Cell Signaling Technology) supplemented with 1.5 Protease Arrest (G-Biosciences, Maryland Heights, MO). Protein concentration was determined using the Pierce BCA protein assay kit (Thermo Scientific). Proteins were separated on 4-20% Mini-Protean TGX Gels (Bio-Rad, Hercules, CA) using 30 µg of total protein per sample. Transfer was done onto nitrocellulose membranes, which were then blocked for 1 hour at room temperature with 2% BSA in PBS. Membranes were rinsed in PBS and then incubated overnight at 4°C in 0.1% TWEEN in PBS containing 2% BSA and primary antibody diluted 1:1000. Membranes were washed the next day with 0.1% TWEEN in PBS every 5 minutes for a total of 30 minutes, then incubated with a 1:10,000 dilution of, either goat anti-rabbit or mouse IgG (Jackson ImmunoResearch, West Grove, PA). Membranes were rinsed a final time before being read on an Odyssey CLx infrared scanner (Li-Cor, Lincoln, Nebraska).

### *TAK1 and JNK Inhibitors*

A TAK1 inhibitor, (5Z)-7-Oxozeaenol, and JNK inhibitor, SP600125, were solubilized in dimethyl sulfoxide (DMSO), and used at working concentrations of 5 µM and 10 µM,

respectively. They were given added to cultures one hour prior to activation with 100ng/mL of IL-33. Supernatants were collected 16 hours later and ELISAs were run to determine cytokine production.

#### *miR-155 qPCR*

BMMCs were cultured in 12.5mM lactic acid for 6 hours. Then, cells were harvested and total RNA was extracted with TRIzol reagent (Life Technologies, Grand Island, NY). cDNA was synthesized using the qScript microRNA cDNA Synthesis Kit (Quantabio, Gaithersburg, MD) following the manufacturer's protocol using oligo dT primers provided in the kit. cDNA was quantified using the Thermo Scientific NanoDrop™ 1000 UV-vis Spectrophotometer (Thermo Scientific, Waltham, MA) according to manufacturer's recommended protocol. qPCR analysis was performed with Bio Rad CFX96 Touch™ Real-Time PCR Detection System (Hercules, CA) and SYBR® Green detection using a relative Livak Method. Each reaction was performed according to the manufacturer's protocol using 8ng of sample cDNA, 12.5µl of PerfeCTa SYBR Green SuperMix (Quantabio, Gaithersburg, MD) and mmu-miR-155 or SNORD47 (housekeeping gene) primers (Quantabio, Gaithersburg, MD) in a final reaction volume of 10 µM. Amplification conditions for all reactions consisted of a heat-activation step at 95 °C for 15 min followed by 40 cycles of 95 °C for 15s, 60 °C for 30s and 70 °C for 15s. Fluorescence data was collected during the extension step of the reaction.

#### *Statistical Analysis*

Data were analyzed using GraphPad Prism 6 software to determine *p* values by Student's *t* test.



## Results

### *Lactic Acid Causes Differential Cytokine Production in C57BL/6 BMMCs Depending on Activation*

Since mast cells in the body can reside in tissues that are rich in lactic acid, we cultured BMMCs in lactic acid rich media and measure the impact lactic acid has on cytokine secretion. Using ELISA, we measured IL-6, TNF, IL-13, MCP-1, and MIP-1 $\alpha$  after IgE crosslinking or IL-33 activation (Figure 1). We found that culturing C57BL/6 BMMCs in 12.5mM lactic acid media for 6 hours prior to IgE crosslinking led to a significant increase IL-6, TNF, MCP-1, and IL-13. By contrast, culturing BMMCs in 12.5mM lactic acid for 24 hours prior to IL-33 activation significantly decreased in production of these cytokines and chemokines.

### *Lactic Acid Causes Differential Cytokine Production in 129/SvJ BMMCs Depending on Activation*

Our lab has recently shown that mice from different backgrounds can have significantly different responses to identical treatments. For example, mast cells from 129/SvJ mice are resistant to the suppressive effects of TGF $\beta$ 1 (25). Therefore, we expanded our investigation to include 129/SvJ BMMC. Using ELISA, we measured IL-6, TNF, MCP-1, IL-13 and MIP-1 $\alpha$  after IgE crosslinking or IL-33 activation (Figure 2). We found that culturing 129/SvJ BMMCs in 12.5mM lactic acid media for 6 hours prior to IgE crosslinking did increases cytokine production as noted with C57BL/6 mice. However, culturing BMMCs in 12.5mM lactic acid for 24 hours prior to IL-33 activation led to a significant decrease in IL-6, TNF, and IL-13. These data suggest that lactic acid effects can vary with both stimuli and genetic background.

### *Lactic Acid Increases VEGF Production and Angiogenesis*

Because lactic acid is a byproduct of hypoxia, and hypoxia induces angiogenesis, investigated lactic acid effects on VEGF production. VEGF was measured by ELISA after 1 hour of culture in 12.5mM lactic acid prior to activation by either IgE crosslinking or IL-33 (Figure 3). We found that lactic acid did not change the amount of VEGF produced by IgE crosslinking on either C57BL/6 or 129/SvJ backgrounds. In contrast to its suppression of IL-33-mediated cytokine and chemokine production, lactic acid significantly increased VEGF secretion from a C57BL/6 BMMC stimulated with IL-33, while having no effect on 129/SvJ BMMC.

### *Lactic Acid Does Not Alter Degranulation*

Since lactic acid significantly increases late phase cytokine secretion during IgE crosslinking, we wondered if lactic acid has the same effect on the early phase response, characterized by degranulation. Cells were cultured in 12.5mM lactic acid for 6 hours prior to IgE crosslinking with DNP-HSA. Measuring the granule markers CD63 and CD107a by cell surface staining, we demonstrate found lactic acid did not change BMMC degranulation (Figure 4).

### *Lactic Acid Arrests BMMCs in G0/G1*

To determine whether lactic acid is toxic to BMMCs, we used PI DNA staining on fixed/permeabilized cells to assess cell cycle progression and the presence of fragmented DNA. Using variable amounts of IL-3 and SCF to induce cell cycle progression, cells were plated in either cRPMI or 12.5mM lactic acid for 36 hours. We discovered that lactic acid diminished the fraction of cells in the S and G2 phases, while increasing the G0/G1 phase. No increase in

apoptotic cells with sub-diploid DNA content was noted, suggesting that lactic acid is not toxic to BMMC under these culture conditions (Figure 5).

#### *Lactic Acid Does Not Change Receptor Expression*

To determine a cause for the differential cytokine production caused by lactic acid, we assessed changes in surface receptor levels using flow cytometry. Cells were cultured in lactic acid for either 6 or 24 hours and then stained for receptors involved in IgE signaling (c-Kit and IgE) and IL-33 signaling (c-Kit and ST2) (Figure 6). The results showed a modest, approximately a 20% decrease, T/ST2 and c-Kit surface expression, and no change in FcεRI levels.

#### *Lactic Acid Effects on IL-33 Cytokine are pH-Dependent, while Effects on IgE are not*

Previous papers demonstrated that pH plays a role in the ability of lactic acid to alter cellular function. To investigate this, we used sodium lactate, the salt of lactic acid does not alter pH when placed in cRPMI media. BMDCs were cultured in 12.5mM lactic acid media or 12.5mM sodium lactate media for 24 hours prior to IL-33 activation. The results showed a clear difference. Lactic acid suppressed IL-33-mediated cytokine secretion as expected, while sodium lactate had no effect (Figure 7).

BMDCs were also cultured in 12.5mM lactic acid media or 12.5mM sodium lactate media for 6 hours prior to IgE crosslinking. We found that sodium lactate was as effective as lactic acid at increasing IgE-mediated MIP-1α secretion (Figure 8). Hence the importance of lactic acid-induced pH change were divergent, depending on the stimulus.

#### *Lactic Acid Decreases Phosphorylation of TAK1 and JNK and Increases AKT Phosphorylation*

We chose to study the mechanism by which lactic acid alters IL-33 signaling, as these changes were greater in magnitude than the effect on IgE signaling. Western blots were performed on cells that were cultured in 12.5mM lactic acid prior to activation with IL-33. After normalization to GAPDH, results showed that phosphorylated JNK (pJNK) and phosphorylated TAK1 (pTAK1) are significantly reduced in lactic acid treated cells vs control BMMC. Phosphorylated AKT (pAkt) was significantly increased in lactic acid treated cells, while phosphorylated p38 (p-p38) and phosphorylated NFκB p65 (p-p65) were not significantly altered (Figure 9).

#### *TAK1 and JNK STIs Are Able to Mimic Lactic Acids Effect on IL-33 Signaling*

The decrease in TAK1 and JNK activation correlated with the ability of lactic acid to reduce IL-33-mediated cytokine secretion. To determine if blocking these pathways alone was sufficient to mimic the effects of lactic acid, we treated BMMC with JNK and TAK1 chemical inhibitors. While the JNK inhibitor had no significant effect, TAK1 inhibition completely extinguished IL-33-mediated cytokine production (Figure 10). Thus, lactic acid effects on TAK1 could be functionally important.

#### *Lactic Acid Decreases Expression of the Pro-Inflammatory Transcript, miR-155*

As part of our search for how lactic acid may be suppressing IL-33-mediated inflammatory signals, we measured expression of miR-155. This microRNA has been widely reported to promote inflammatory responses by controlling expression of many genes (28-31). As shown in Figure 11, we found that lactic acid treatment for 6 hours decreased miR-155 expression by more than 50%, as measured by quantitative PCR. These data suggest lactic acid could have broad effects on mast cell function, by controlling this microRNA.

## Discussion

Lactic acid is a byproduct of anaerobic glycolysis and is known to be increased in a variety of diseases such as cancer, obesity, Type II diabetes and wound healing (17, 21). While lactic acid has been shown to inhibit cytotoxic T cells ability to kill, promote M2 macrophage differentiation, and prevent dendritic cell antigen presentation, its effects on mast cell function have not yet been investigated (12, 14, 17). Mast cells are known to participate in allergic disease, parasitic infections, and resistance to bacteria. They have less defined roles in cancer and wound healing, but are known to participate in both (26, 32-35). Our data indicate that lactic acid alters inflammatory cytokine production in mast cells in a stimuli-dependent manner. In the context of cancer, this could lead to cancerous cells escaping immune surveillance. For wound healing, it could help strike the balance between a pro-resolving environment or allow for a chronic wound environment due to the inability to clear invading pathogens.

C57BL/6 mice exhibit increased cytokine production when exposed to lactic acid prior to IgE crosslinking, and exhibit decreased cytokine production when exposed to lactic acid prior to IL-33 activation. 129/SvJ mice exhibit the same inhibition of cytokine production when activated with IL-33. However, during IgE crosslinking with DNP-HSA, lactic acid does not increase cytokine production in 129/SvJ mice as seen in the C57BL/6 BMMC. Differential responses between these two genetic backgrounds have been demonstrated before by our group and others (25, 36-38). These variations have potential clinical relevance, since human populations show varied tendencies to develop inflammatory diseases and varied therapeutic responses because of genetic variation. C57BL/6 and 129/SvJ mice are known to differ in their default immune responses, with C57BL/6 mice mounting strong TH1 responses, and 129/Sv having strong TH2

responses. 129/SvJ mice are also more prone to autoimmunity (36). The reasons for their differential responses could be due to polymorphisms, such as those noted by Ford et al. in the CD23 sequence. Unraveling the mechanisms by which lactic acid alters mast cell function will provide targets for studying this difference.

Based on previous work involving lactic acid, we also determined that the change in IL-33 signaling is related with the change in pH (14). Sodium lactate is the salt of lactic acid, with the carboxyl group being replaced with an ionic bond to sodium. This results in sodium lactate not being able to donate a proton into solution and thus no pH change accompanies the addition of sodium lactate into media. Our results show that without the change in pH, cytokine and chemokine production induced by IL-33 were unaltered. Interestingly, the increase in cytokine and chemokine production that lactic acid causes during IgE crosslinking is not consistent when lactic acid or sodium lactate is added to BMMC cultures. This indicates that pH is not involved in the increase of IgE mediated cytokines and chemokines, and instead that the lactate structure itself is somehow driving the change. This warrants further investigation. Asthma leads to increased lactic acid and IL-33 production (39, 40).

PI-DNA staining revealed that lactic acid did not elicit cell death in our culture conditions. However, lactic acid did appear to suppress proliferation, as shown by a slight but consistent increase in G0/G1 phase, matched by a 50% reduction in the S and G2 phases of the cell cycle. Lastly, we noted that in the absence of growth factors, BMMC cultured in lactic acid retained approximately 30% of the culture in the live, G0/G1 phase, compared to less than 10% of cells cultured without lactic acid. We hypothesize that this is related to the increase in Akt expression we noted in Western blots, as Akt promotes survival (41, 42).

Mast cells are known to secrete VEGF and participate in angiogenesis. Lactic acid has also been shown to induce VEGF production in macrophages (19). However, to our knowledge this has not been demonstrated in mast cells until now. Our data show that within an hour of lactic acid exposure, VEGF production significantly increased when following IL-33 stimulation. Combining this with the need for neovasculature needed for tumors to grow and wounds to heal, it appears as if the microenvironment is dictating the phenotype of the mast cell by using pH and hypoxia. Functional studies need to be carried out to test this hypothesis.

Lactate has been shown to increase NFκB signaling in macrophages stimulated with LPS (17, 43). We investigated the mechanism by which lactic acid altered IL-33 signaling using western blots. IL-33 is known to activate the TAK1 MAP3K as an apical kinase, with resulting downstream activation of NFκB and JNK/p38 in mast cells (44). Our data show that while p38 and NFκB p65 phosphorylation is not affected, TAK1 and JNK phosphorylation was significantly decreased and correlated with the decreased cytokine production seen in Figures 1 and 2. The TAK1 inhibitor, (5Z)-7-Oxozeaenol completely ablated IL-6 and IL-13 production in response to IL-33, while chemical blockade of JNK had no effect. We therefore postulate that reduced TAK1 activation may be the critical mechanism by which lactic acid suppresses IL-33 signaling.

In conclusion, the lactic acid-rich environment associated with TME and wound healing affect mast cell-mediated inflammation in a stimulus and genetic background dependent manner. IL-33 activation in the presence of lactic acid gives an anti-inflammatory and pro-angiogenic response, while IgE crosslinking enhances cytokine and chemokine production. While further investigation is needed for more detailed mechanistic data as well as functional *in vivo*

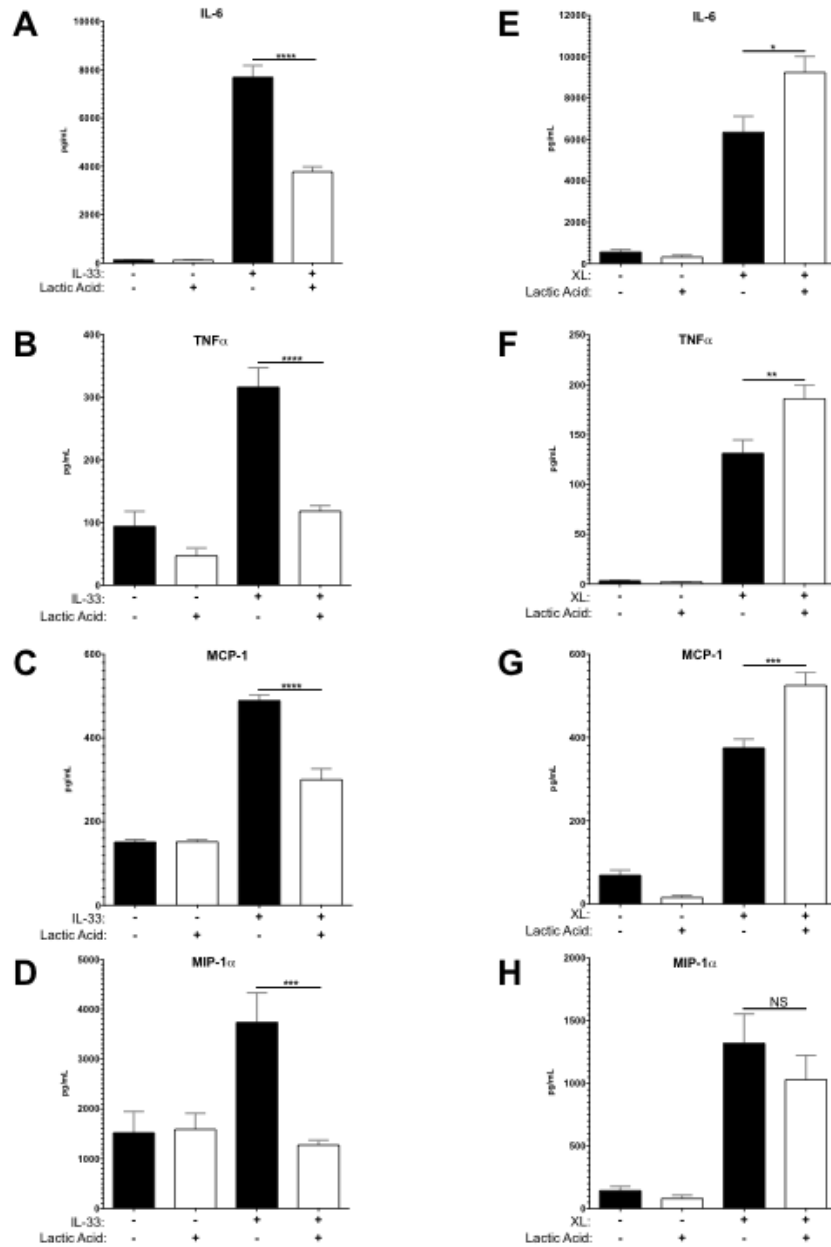
experiments, this provides a potential starting point for developing therapies to mitigate inflammation in mast cell-dependent diseases.



## **FIGURES**

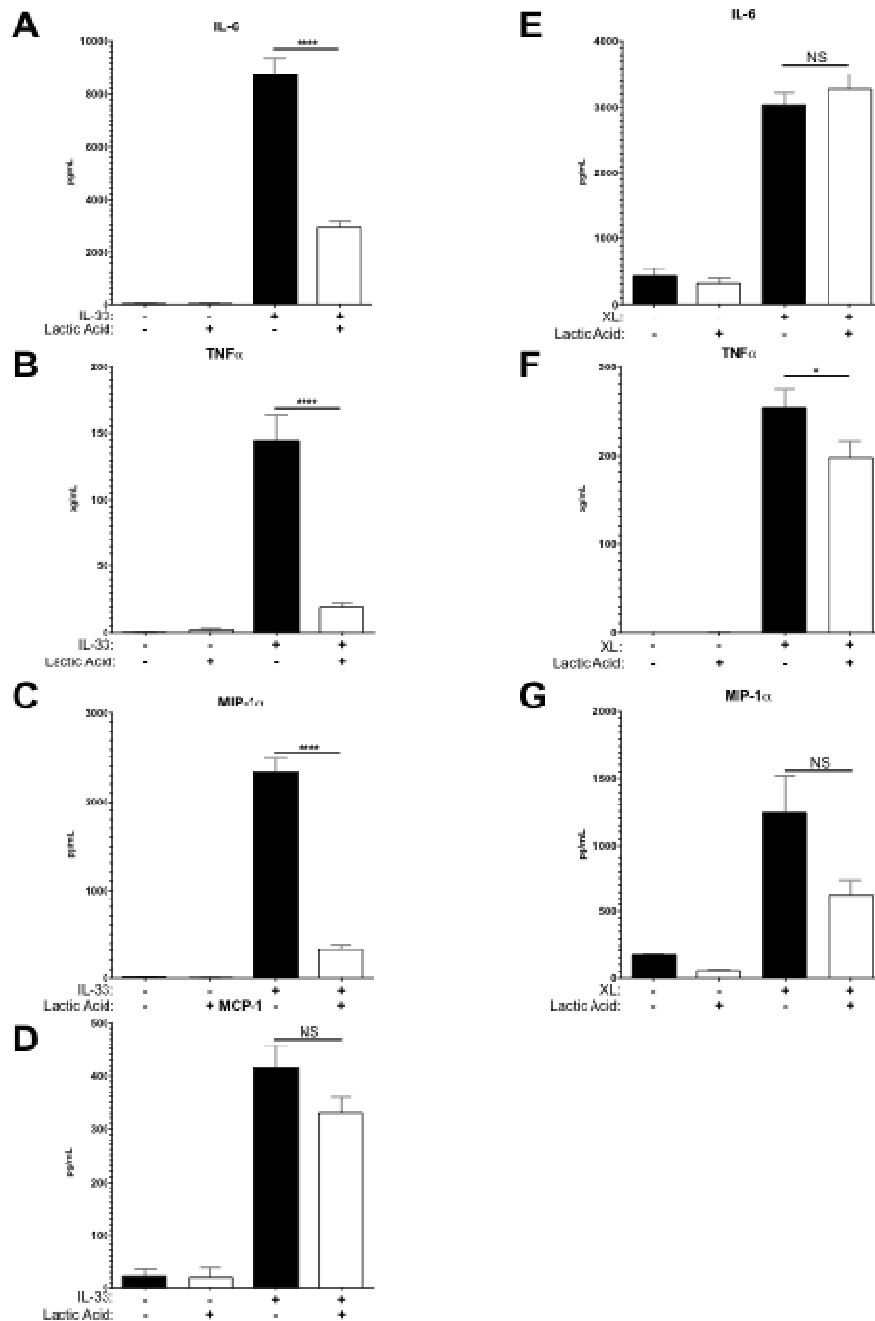
**Figure 1A-H**

**Figure 1:** ELISAs were performed on BMMC supernatants from C57BL/6 mice that were activated by either IgE crosslinking or by IL-33 stimulation. IgE crosslinked cells were exposed to 12.5mM lactic acid media for 6 hours prior to crosslinking. Cells that were activated with IL-33 were exposed to lactic acid for 24 hours prior to activation. A. IL-6 ELISA. B. TNF ELISA. C. MCP-1 ELISA. D. MIP-1 $\alpha$  ELISA. E. IL-6 ELISA. F. TNF ELISA. G. MCP-1 ELISA. H. MIP-1 $\alpha$  ELISA. Data shown are representative of at least 3 experiments with n=3 for each experiment. \*  $p<0.05$ , \*\*  $p<0.01$ , \*\*\*  $p<0.0005$ , \*\*\*\*  $p<0.000$



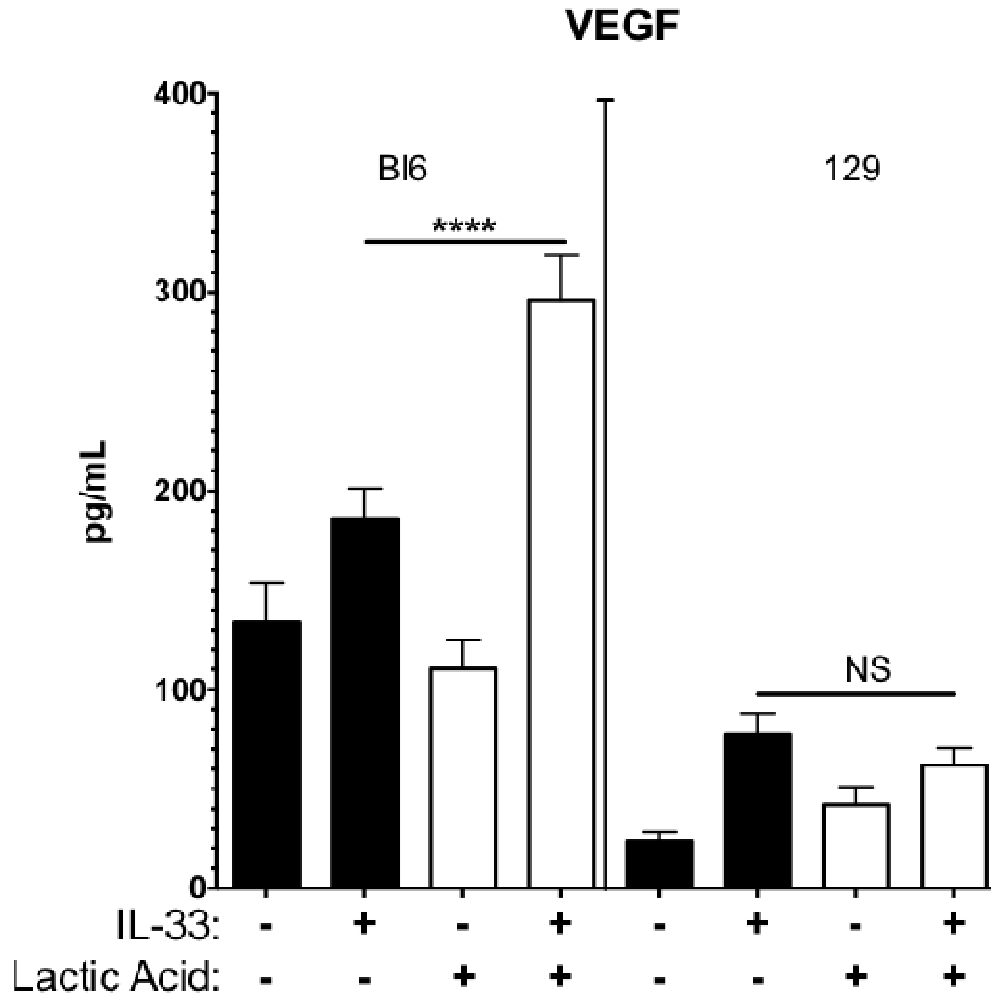
**Figure 2A-G**

**Figure 2:** ELISAs were performed on BMMC supernatants from 129/SvJ mice that were activated by either IgE crosslinking or by IL-33 stimulation. IgE crosslinked cells were exposed to 12.5mM lactic acid media for 6 hours prior to crosslinking. Cells that were activated with IL-33 were exposed to lactic acid for 24 hours prior to activation. A. IL-6 ELISA. B. TNF ELISA. C. MIP-1  $\alpha$  ELISA. D. MCP-1 ELISA. E. IL-6 ELISA. F. TNF ELISA G. MCP-1 ELISA. Data shown are representative of at least 3 experiments with n=3 for each experiment. \*  $p<0.05$ , \*\*  $p<0.01$ , \*\*\*  $p<0.0005$ , \*\*\*\*  $p<0.0001$



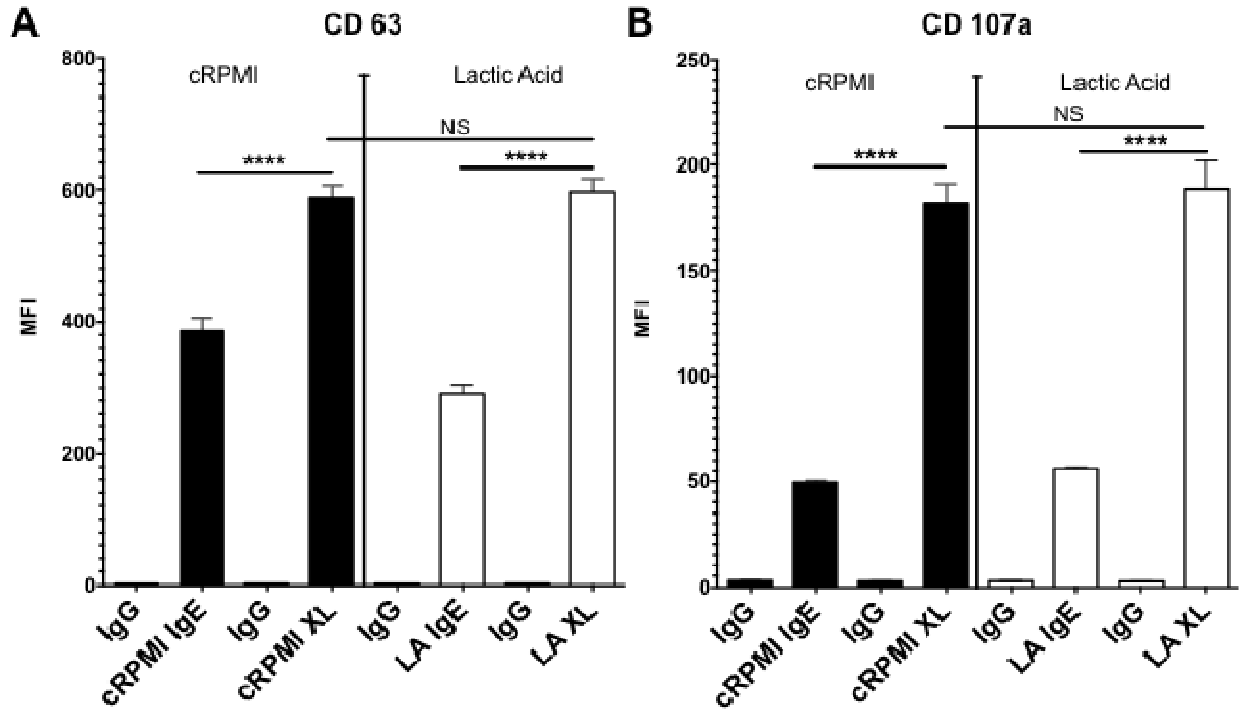
**Figure 3A-B**

**Figure 3:** VEGF ELISAs on BMMC supernatants C57C57BL/6 and 129/SvJ mice that were activated by IL-33 stimulation. Data shown are representative of at least 3 experiments with n=3 for each experiment. \*  $p<0.05$ , \*\*  $p<0.01$ , \*\*\*  $p<0.0005$ , \*\*\*\*  $p<0.0001$



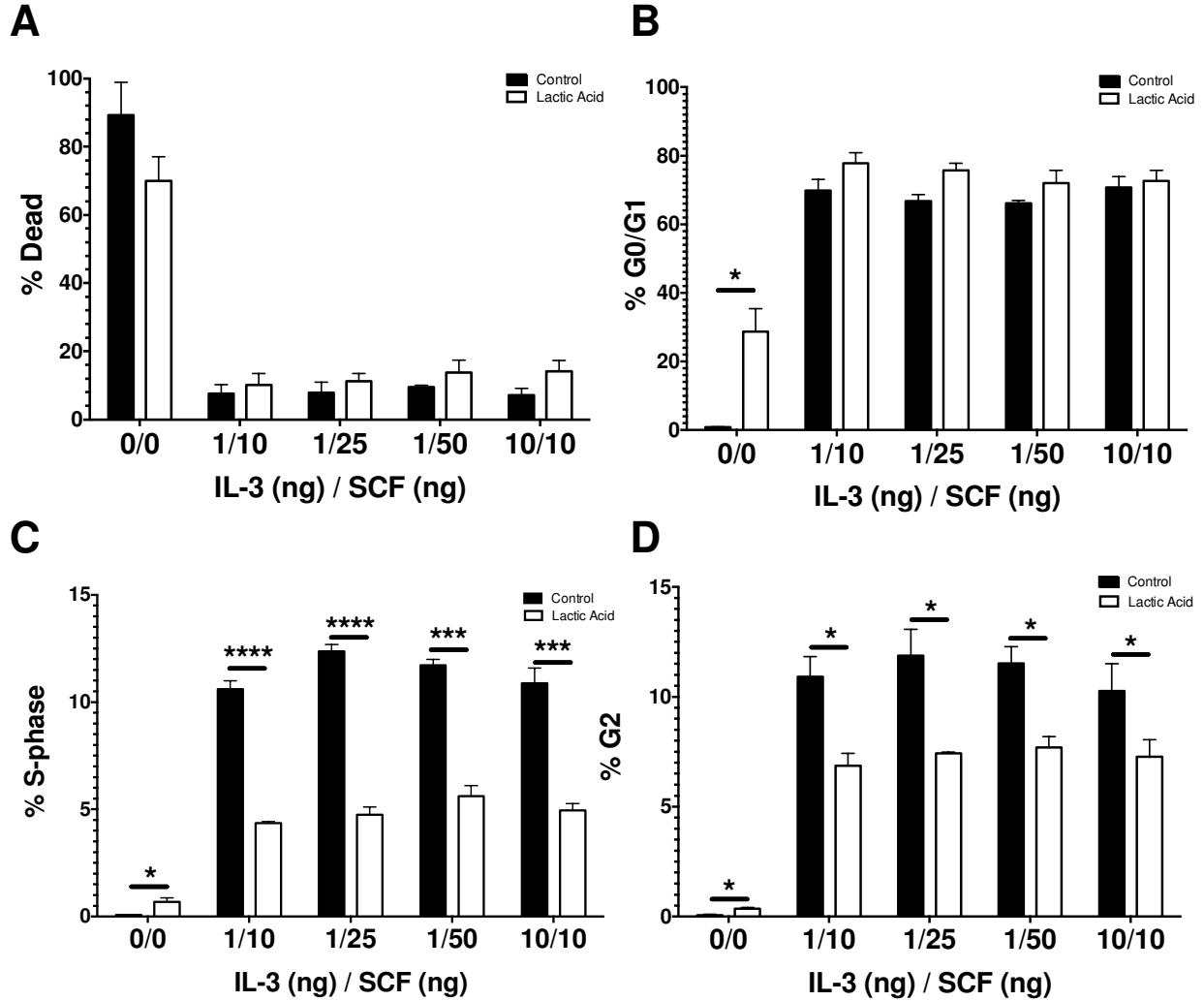
**Figure 4A-B**

**Figure 4:** C57BL/6 BMMCs were checked to see if lactic acid affected degranulation during IgE cross linking using two different surface markers, CD63 and CD107A. A) CD 63. B) CD107a. Data shown are representative of at least 3 experiments with n=3 for each experiment. \*  $p<0.05$ , \*\*  $p<0.01$ , \*\*\*  $p<0.0005$ , \*\*\*\*  $p<0.0001$



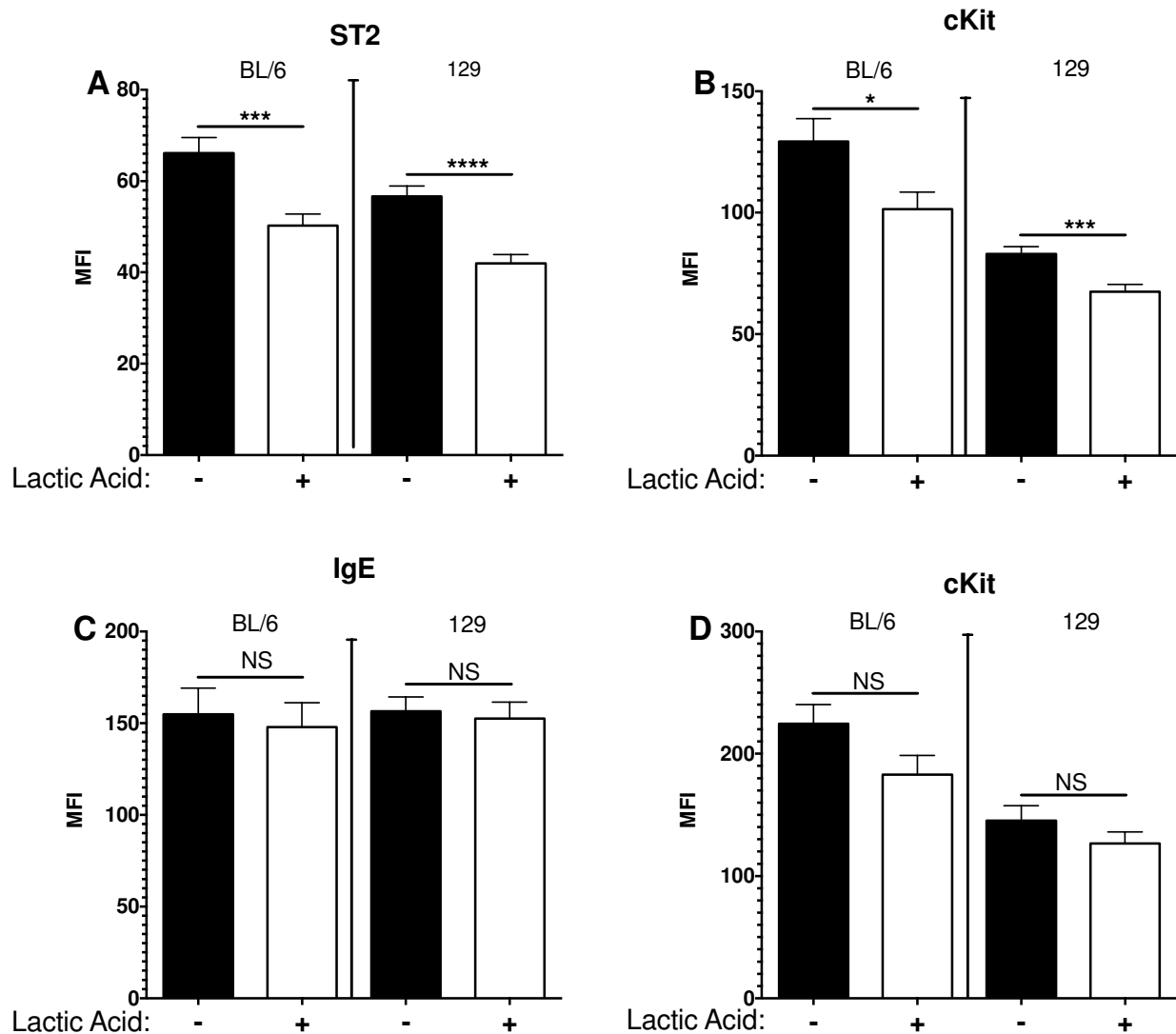
**Figure 5A-D**

**Figure 5:** BMMC were cultured in variable amounts of IL-3 and SCF in the presence or absence of lactic acid for 36 hours, after which PI DNA staining was run on flow cytometry to determine cell phase progression. A. Cell death. B. G0/G1 phase. C. S phase. D. G2 phase. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.0005$ , \*\*\*\*  $p < 0.0001$



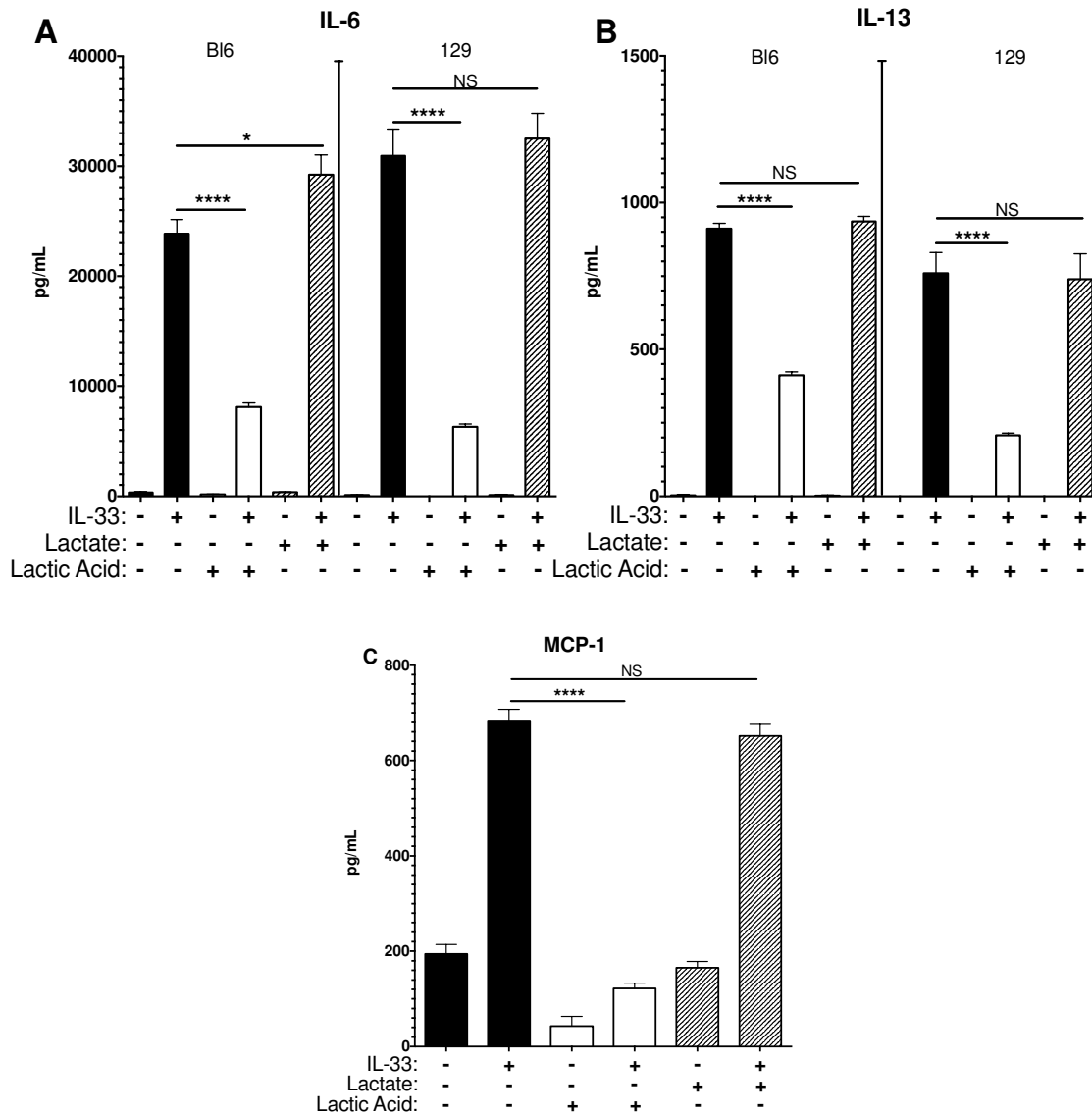
**Figure 6A-D**

**Figure 6:** BMMCs were checked by flow cytometry for changes in receptor expression in response to lactic acid pretreatment. Cells were pretreated in lactic acid for either 6 or 24 hours prior to receptor staining. A. MFI of T1/ST2 after 24 hours of lactic acid treatment. B. MFI of c-kit after 24 hours of lactic acid treatment. C. MFI of anti-IgE after 24 hours lactic acid treatment. D. MFI of c-Kit after 6 hours lactic acid treatment. Data shown are representative of at least 3 experiments with n=3 for each experiment. \*  $p<0.05$ , \*\*  $p<0.01$ , \*\*\*  $p<0.0005$ , \*\*\*\*  $p<0.0001$



**Figure 7A-C**

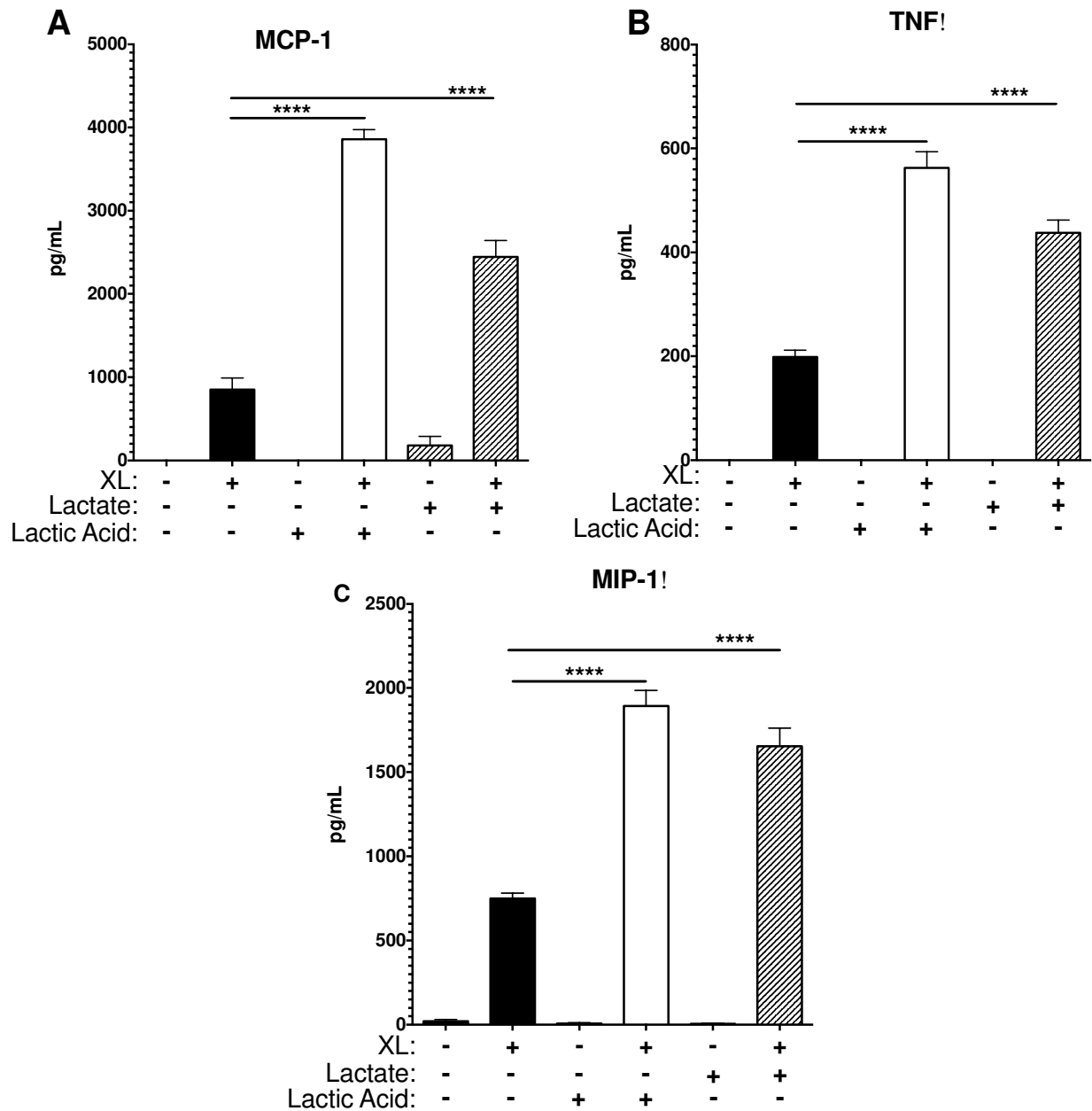
**Figure 7:** BMMCs were given sodium lactate to determine if the decrease in cytokine production during IL-3 stimulation was related to pH. BMMCs were in lactic acid or sodium lactate media for 24 hours prior to IL-33 activation. A) IL-6 B) IL-13 C) MCP-1. Data shown are representative of at least 2 experiments with n=3 for each experiment. \*  $p<0.05$ , \*\*  $p<0.01$ , \*\*\*  $p<0.0005$ , \*\*\*\*  $p<0.0001$





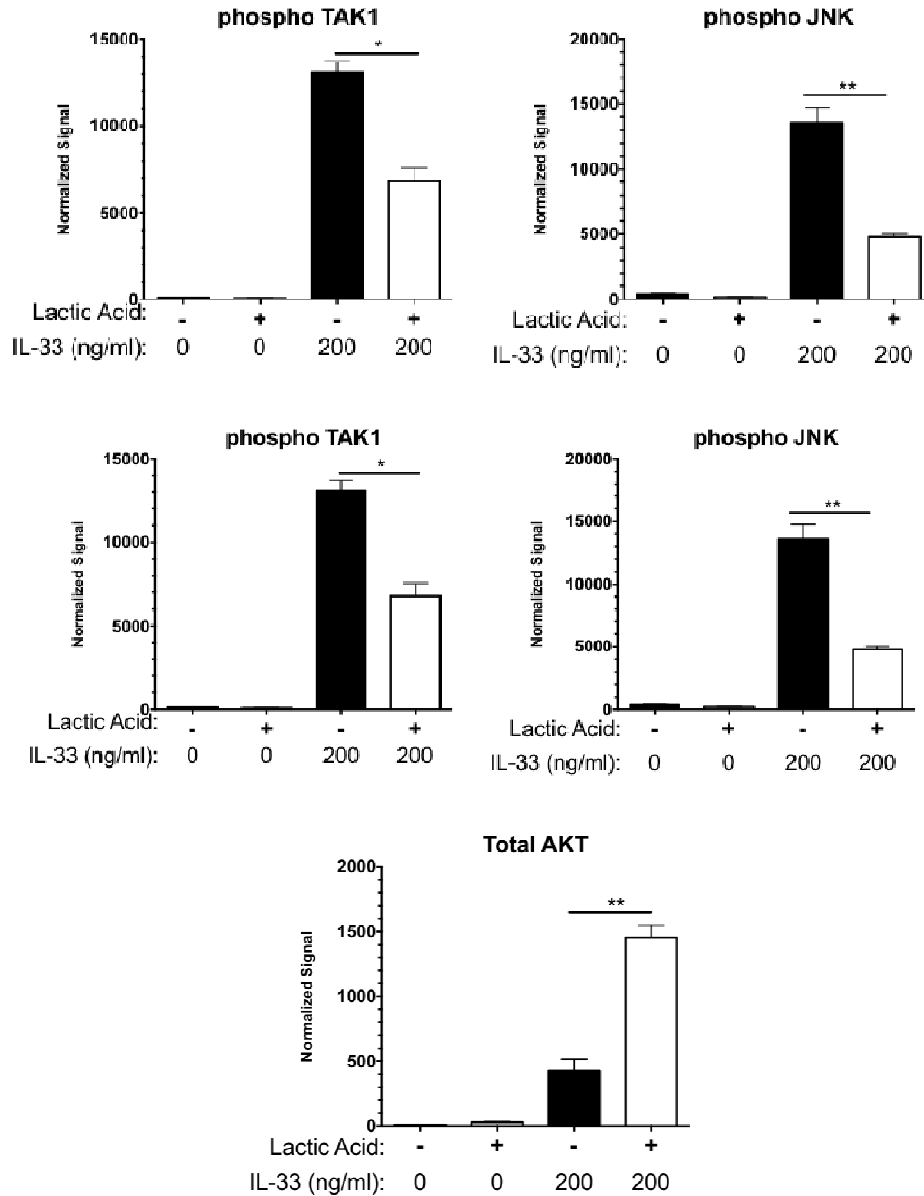
**Figure 8A-C**

**Figure 8:** BMMCs were given sodium lactate to determine if the increase in cytokine production during IgE crosslinking was due to pH. BMMCs were in lactic acid or sodium lactate media for 6 hours prior to IgE crosslinking with DNP-HSA. A) MCP-1 B) TNF C) MIP-1 $\alpha$ . Data shown are representative of at least 3 experiments with n=3 for each experiment. \*  $p<0.05$ , \*\*  $p<0.01$ , \*\*\*  $p<0.0005$ , \*\*\*\*  $p<0.0001$



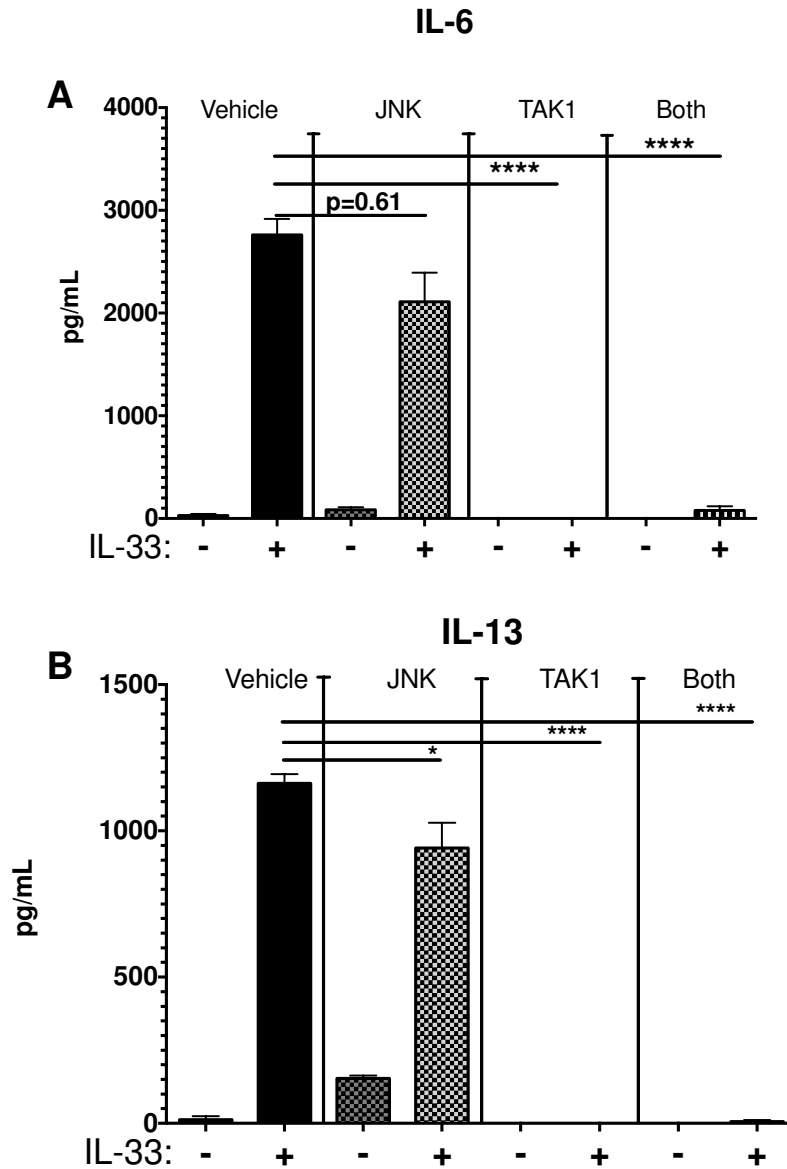
**Figure 9A-E**

**Figure 9:** Western Blots were run on C57BL/6 mice to determine the change lactic acid was causing in the IL-33 signaling pathway. 15 minutes of IL-33 activation is shown as signal normalized to the loading control. A) Phospho-TAK1 B) Phospho-JNK C) Phospho-p65 D) Phospho-p38 E) Phospho-AKT. \*  $p<0.05$ , \*\*  $p<0.01$ , \*\*\*  $p<0.0005$ , \*\*\*\*  $p<0.0001$



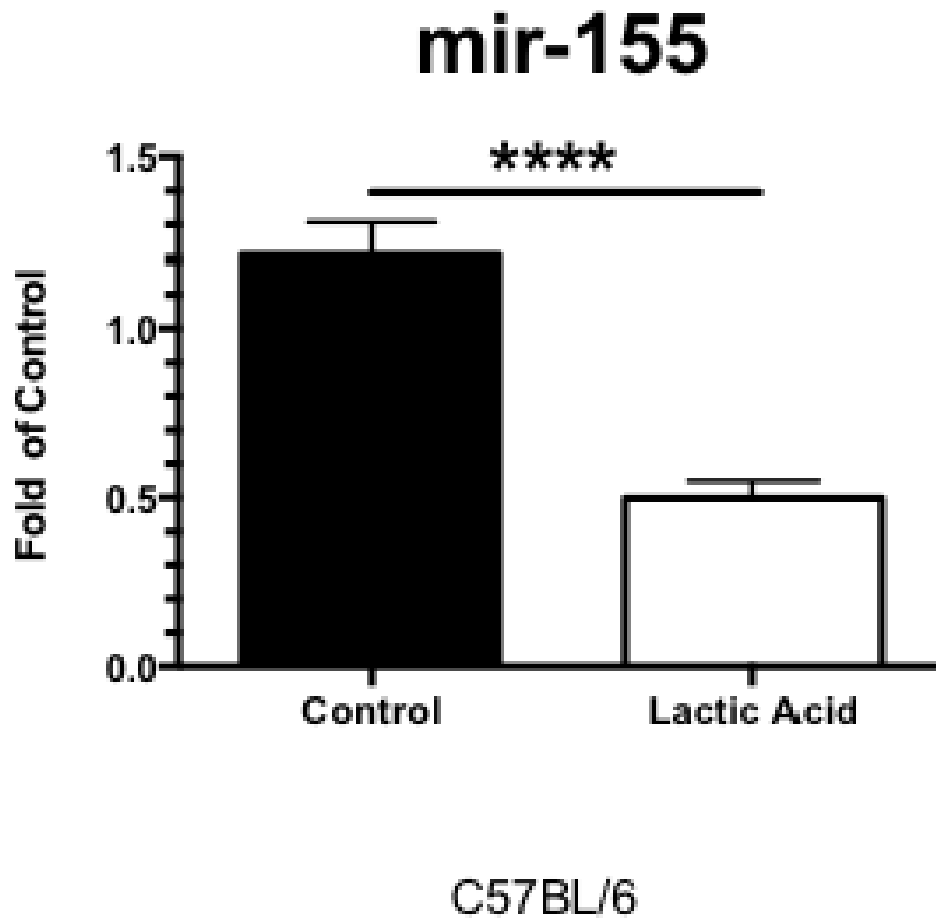
**Figure 10A-B**

**Figure 10:** TAK1 and JNK inhibitors were given to determine if they could mimic the effects of lactic acid on IL-33 signaling. A) IL-6 B) IL-13. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.0005$ , \*\*\*\*  $p < 0.0001$



**Figure 11**

**Figure 11:** qPCR on mir155. Data shown are representative of at least 3 experiments with n=3 for each experiment. \*  $p<0.05$ , \*\*  $p<0.01$ , \*\*\*  $p<0.0005$ , \*\*\*\*  $p<0.0001$



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## **Vita**

Andrew Spence was born in Midlothian, Virginia, to Catherine and Okema Spence, on May 23, 1990. He attended Virginia Commonwealth University and graduated in 2012 with a Bachelor of Science in Biology. He worked in the Bowlin Tissue Engineering Lab before beginning his M.S studies in the Ryan Lab. During his M.S. studies, he taught introductory biology lab courses, took classes, and carried out many lactic acid experiments. He has to date published four peer-reviewed articles. He lives with his brother, Michael Spence, friend Andy “Playoff Bound” Hobson, and two dogs, Remington Steel and Spencer Spence.